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To identify novel components that affect the ER transcriptional response, we performed a genetic screen in yeast and identified RDI1, a Rho guanine nucleotide dissociation inhibitor, as a positive regulator of ER transactivation. In contrast, expression of constitutively active forms of RhoA, Rac1, and Cdc42 decreases ER transcriptional activity, suggesting that Rho GDI increases ER transactivation by antagonizing ER inhibition by Rho GTPases. Our recent results indicate that the Rho GDI signal is transduced to ER by CBP/p300 through GRIP1-dependent and -independent pathways. Together, these findings establish Rho GTPases as important modulators of ER transcriptional activation by regulating of GRIP1 and CPB coactivator activity. Our data suggest a complex relationship between ER transactivation and the Rho signaling pathways through modulation of receptor cofactors, which may have evolved to coordinate receptor-dependent gene expression with Rho-regulated events, such as cell migration. Our results also suggest that dysregulation of the Rho-ER axis may participate in cancer progression.

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Introduction:

Estrogens play a key role in the development of breast cancer. Estrogens activate an intracellular protein, termed the estrogen receptor (ER), which acts in concert with certain other proteins to "switch on" some genes and "switch off' others that lead to breast cancer growth. The removal of estrogen through the use of inhibitors, such as tamoxifen, results in regression of most breast cancers. Unfortunately, this effect is often short-lived, with breast cancer cells reappearing that are estrogen-independent for growth. Since estrogen-independent tumors often still express the ER, it has been proposed that the ER has "learned" to use other factors to activate cell growth in the absence of estrogen. Once this happens, the tumor becomes much more difficult to eradicate with the current treatment options. The goal of this project is to identify new factors critical for ER function and, thereby, identify potential targets for future breast cancer therapy.

Body:

Estrogen receptor alpha (ERα) is a steroid hormone receptor that coordinates gene expression with cellular physiology in response to extracellular signals. While signaling pathways that modulate ER and cofactor activities have been described, much remain unknown between the interaction of the complex web of cellular signaling with ER transcriptional activity. To identify novel components that affect the ER transcriptional response, we performed a genetic screen in yeast and identified RDI1, a Rho guanine nucleotide dissociation inhibitor, as a positive regulator of ER transactivation. Overexpression of the human homologue, Rho GDIα, specifically increases the ER, androgen receptor and glucocorticoid transcriptional activation in mammalian cells. In contrast, expression of constitutively active forms of RhoA, Rac1, and Cdc42 decreases ER transcriptional activity, suggesting that Rho GDI increases ER transactivation by antagonizing ER inhibition by Rho GTPases. Other means of inhibiting RhoA by expression of either the Clostridium botulinum C3 transferase or a dominant negative RhoA also resulted in enhanced ER transcriptional activation, thus phenocopying the effect of Rho GDI expression on ER transactivation. Together, these findings establish Rho GTPases as important modulators of ER transcriptional activation.

Further characterization of Rho signaling pathway indicate that Rho GDI enhances ER transactivation by increasing the activity of ER activating proteins termed coactivators that promote basal transcriptional factor assembly. We show that Rho GDI cooperates with the coactivator GRIP1 to increase ER ligand-independent and ligand-dependent transcriptional activation, and also enhances GRIP1 transcriptional activity when GRIP1 is tethered to DNA. The GRIP1activation domain 1 (AD1), which binds CBP/p300, is necessary for Rho GDI to modulate GRIP1 activity. Using E1A to inhibit the endogenous CBP/p300 and a Gal4-CBP fusion protein to assay CBP activity, we find that the effect of

Rho GDI on ER transcriptional activation is CBP/p300-dependent. Importantly, the ability of CBP/p300 to transduce the Rho GDI signal occurs through both GRIP1-dependent and -independent pathways. These data suggest a complex interplay between ER transcriptional regulation and the Rho signaling pathways through modulation of receptor cofactors, which may have evolved to coordinate receptor-dependent gene expression with Rho-regulated events, such as cell migration.

Key Research Accomplishments

- Identified Rho GDI as a modulator of ER transcriptional activity
- Rho GDI increase ER transactivation by blocking ER inhibition by Rho GTPases
- Rho signaling pathway modulate ER by regulating the activities of the coactivators, GRIP1 and CBP
- Rho GDI synergizes with GRIP1 to stimulate ER transactivation
- Synergistic ER activation by Rho GDI and GRIP1 is dependent on both N- and C-terminal transactivation domains on ER

Reportable outcome

Su, L. F., Knoblauch, R., Garabedian, M. J. *Rho GTPases as modulators of the estrogen receptor transcriptional response.* J Biol Chem, 2001. 276(5): p. 3231-3237

Su, L. F. Wong, Z., Garabedian, M. J. *Regulation of GRIP1 and CPB coactivator activity by Rho GDI modulates estrogen receptor transcriptional enhancement.* J Biol Chem, 2002. (submitted).

Conclusion

We have, through an unique screening process, identified Rho GDI as a protein that increases ER transcriptional activity when overexpressed. Rho GDI has been shown previously to regulate both cell shape and movement, the acquisition of which are important for invasion and spreading of breast tumor cells. The identification of Rho GDI as an effector of ER transcriptional regulation provides a link between changes in cellular architecture and ER activity. The identification of such a connection may offer a potential new strategy for turning off ER-dependent transcription activation, and, thereby, shutting off breast cancer growth.

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Rho GTPases as Modulators of the Estrogen Receptor Transcriptional Response*

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The estrogen receptor α (ER) is a ligand-dependent transcription factor that plays a critical role in the development and progression of breast cancer, in part, by regulating target genes involved in cellular proliferation. To identify novel components that affect the ER transcriptional response, we performed a genetic screen in yeast and identified RDI1, a Rho guanine nucleotide dissociation inhibitor (Rho GDI), as a positive regulator of ER transactivation. Overexpression of the human homologue of RDI1, Rho GDIα, increases ERα, ERβ, androgen receptor, and glucocorticoid receptor transcriptional activation in mammalian cells but not activation by the unrelated transcription factors serum response factor and Sp1. In contrast, expression of constitutively active forms of RhoA, Rac1, and Cdc42 decrease ER transcriptional activity, suggesting that Rho GDI increases ER transactivation by antagonizing Rho function. Inhibition of RhoA by expression of either the *Clostridium botulinum* C3 transferase or a dominant negative RhoA resulted in enhanced ER transcriptional activation, thus phenocopying the effect of Rho GDI expression on ER transactivation. Together, these findings establish the Rho GTPases as important modulators of ER transcriptional activation. Since Rho GTPases regulate actin polymerization, our findings suggest a link between the major regulators of cellular architecture and steroid receptor transcriptional response.

The estrogen receptor α (ER)[1] is a ligand-dependent transcription factor that transduces the estrogen signal (1). Activation of ER is responsible for female sexual development and maintenance of bone density (2, 3). In addition, ER plays a critical role in the development and progression of breast cancer by regulating genes and signaling pathways involved in cellular proliferation (4). Regulation of gene expression by the ER requires the coordinate activity of ligand binding, phosphorylation, and cofactor interactions, with particular combinations probably resulting in the tissue-specific responses elicited by the receptor (5–7). However, the extracellular cues and intracellular signaling pathways modulating these components and regulating ER transcriptional activation are not fully understood.

To identify novel proteins that modulate ER transcriptional activation, we have carried out a genetic screen in the yeast *Saccharomyces cerevisiae*. The ability of the human ER to function within yeast allows a wide variety of genetic approaches to be taken toward further defining the mechanism of ER transcriptional activation given the ease of genetic manipulation and simplicity of gene identification in yeast. In addition, with the large number of orthologous proteins carrying out the same biological functions in both *S. cerevisiae* and metazoans (8–10), it is likely that the yeast factors affecting ER transactivation will have mammalian counterparts, which can be examined in vertebrate systems.

The genetic approach we have used to identify factors that affect ER transcriptional activation is dosage suppression analysis. In this technique, a mutant ER protein with a reduced ability to activate transcription is used as a substrate to isolate yeast gene product(s) that are capable of overcoming this mutant phenotype, thus restoring receptor transcriptional activity. The mutant ER derivative used in this screen is defective in transactivation by virtue of serine to alanine mutations in the three major N-terminal phosphorylation sites, serines 104, 106, and 118 (ER_{AAA}). This mutant was selected as the substrate because it has only a modest effect on ER transactivation and therefore has the potential to isolate a broad range of factors that affect receptor activity. We expect to isolate yeast factors that enhance ER transcriptional activity and, importantly, have human homologues that can then be examined in mammalian cells for effects on ER transcriptional response. This approach has proven successful for investigating various aspects of ER signal transduction (11). Using this system, we have isolated RDI1, the yeast Rho guanine nucleotide dissociation inhibitor (Rho GDI), as a gene product that is capable of increasing both ER_{AAA} and WT ER transcriptional activation when overexpressed. This gene product is the yeast homologue of the mammalian Rho GDIα, a cytoplasmic protein originally identified as a negative regulator of the Rho family of GTP-binding proteins (12–15). The Rho family of GTPases, which include RhoA, Rac1, and Cdc42, are best known for their ability to regulate actin cytoskeletal remodeling in response to extracellular signals, thereby promoting changes in cell morphology, adhesion, and motility (16). In addition, by affecting multiple signaling pathways, Rho family members regulate gene transcription and cell cycle progression and have been implicated in

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[1] The abbreviations used are: ER or ERα, estrogen receptor α; ERβ, estrogen receptor β; GDI, guanine nucleotide dissociation inhibitor; WT, wild type; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; GR, glucocorticoid receptor; AR, androgen receptor; SRF, serum response factor; ERE, estrogen response element; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; RLU, relative luminescence units.

cellular transformation and metastasis (17–22). The Rho family members possess intrinsic GTPase activity and cycle between the inactive cytoplasmic GDP-bound and the active membrane-associated GTP-bound state. The exchange of GDP for GTP induces a conformational change in the G protein that allows effector molecules, such as protein kinases, to bind and initiate downstream signaling events (23). This GTP/GDP cycle is tightly regulated in response to extracellular signals by three different classes of proteins. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, GTPase-activating proteins (GAPs) accelerate the intrinsic GTPase activity of the Rho GTPases, and GDIs antagonize their activity by blocking GEFs and GAPs (12, 24). However, since the cytoplasmic GDP-bound Rho GTPases predominate under physiological conditions, Rho GDI acts as a negative regulator of Rho GTPases mainly by blocking the dissociation of GDP. In addition, Rho GDI controls the subcellular localization of the GTPases, stimulating their release from the plasma membrane (12, 25). Interestingly, Rubino *et al.* (26) identified an ER-interacting protein, termed Brx, which contains a domain virtually identical to the Rho GEF Lbc and was first to demonstrate a link between ER signaling and the Rho GTPases.

In this report, we extend the findings of Rubino *et al.* (26) and examine the effects of human Rho GDIα as well as the Rho GTPases, RhoA, Rac1, Cdc42, on transcriptional activation by ER and other members of the steroid receptor family in mammalian cells. Our findings indicate that Rho GDIα specifically increases the transcriptional activity of ERα and ERβ as well as the glucocorticoid receptor (GR) and androgen receptor (AR), but not of the unrelated transcription factors serum response factor (SRF) and Sp1, and that this activation is mediated via repression of Rho GTPases. These results further establish the Rho-mediated signaling pathway as an important regulator of ER, GR, and AR transcriptional activity.

EXPERIMENTAL PROCEDURES

Plasmids

Yeast—The reporter plasmid ERE-CYC1-LacZ contains a single estrogen response element (ERE) upstream of a truncated *CYC1* promoter linked to the β-galactosidase gene (27). The yeast high copy genomic library was described by Engebrecht *et al.* (28) and was generated by subcloning *Sau*3A partially digested yeast genomic DNA into the *Bam*HI site of the YEP351 plasmid. WT ER and ER_{AAA} were expressed from the Gal1–10 promoter in *Trp1*, 2 μM plasmid (p2T-GAL) (29). p2T-GAL-ER_{AAA} was constructed by subcloning the *Bam*HI fragment containing ER_{AAA} sequence from pcDNA3 (30) plasmid into p2T-GAL (29).

Mammalian Cells—The ER reporter plasmid contains one ERE from the *Xenopus* vitellogenin A2 gene, upstream of the herpes simplex virus thymidine kinase promoter (−109) linked to the firefly luciferase coding sequence (XETL) (31). The GR reporter plasmid ($XG_{46}TL$) is identical to XETL, except two consensus GREs are substituted for the ERE (31). The steroid receptor expression plasmids are pcDNA3-human ERα, pCMV5-human ERβ (32), pcDNA3-rat GR (33), and pcDNA3-human AR (34). A *Bam*HI/*Eco*RI fragment of human Rho GDIα from pGEX2T (35) was subcloned into pcDNA3 to create a Rho GDIα mammalian expression construct.[2] Expression plasmids for N-terminally Myc-tagged Rac1.L61 and Cdc42.L61 have been described previously (36). pRK5-Myc-RhoA.V14 was subcloned as an *Eco*RI fragment containing Myc-tagged RhoA.V14 from EXV plasmid (18). Both EXV.RhoA.V14 and EFC3- expressing Myc-tagged C3 transferase under the EF1α promoter have been described elsewhere (37, 38). The dominant negative form of RhoA, RhoA.N19, was made by site-directed mutagenesis using the oligonucleotide 5′-GGAGCCTGTGGAAAGAACTGCTTGCTCATAGTC-3′ and the QuickChange mutagenesis kit (Stratagene) with pRK5-Myc-RhoA as the template. The entire RhoA.N19 coding region was sequenced to verify the base changed and to ensure that no other mutations were introduced. The Sp1 reporter contains six Sp1 binding sites upstream of the adenovirus major late promoter in front of the luciferase gene (39), and SRF reporter contains a fragment of the c-Fos promoter upstream of luciferase (18).

Yeast Strains, Growth Conditions, and β-Galactosidase Assay

The yeast strain W303a (**a** *ade2 leu2 his3 trp1 ura3*) was used to screen for ER activators. Yeast transformation was performed by the lithium acetate/polyethylene glycol method (40). To assay ER transcriptional activation, cells were cultured overnight in the appropriate selective medium containing 2% glucose and subcultured 1:20 in selective minimal medium containing 2% galactose, 1% raffinose to induce receptor expression and treated with 17β-estradiol for 12 h. Quantitative liquid β-galactosidase assays were performed as described previously (11). Plate assays were performed by replica-plating colonies from glucose plates onto galactose X-gal indicator plates containing 0.1 nM 17β-estradiol.

Cell Culture, Transfection, and Luciferase Assays

Human osteosarcoma U2OS (HTB 96) and human breast cancer MCF-7 (HTB-22) cell lines were obtained from the American Type Culture Collection (Manassas, VA), and the Ishikawa human uterine cancer cell line was obtained from Dr. Seth Guller (NYU School of Medicine). Cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone), 10 units/ml each of penicillin and streptomycin (Cellgro), and 2 mM L-glutamine (Cellgro). Between 1.2 and 1.3 × 10^5 cells were seeded onto 35-mm plates in phenol red-free Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% charcoal-stripped fetal bovine serum and 2 mM L-glutamine. Transfections using LipofectAMINE Plus reagent (Life Technologies) were performed according to the manufacturer's recommendation. Cells were treated with hormone agonists (100 nM 17β-estradiol, 100 nM dexamethasone, and 100 nM R1881 for ER, GR, and AR, respectively), the ER antagonist ICI 182,780 (41) (100 nM), or ethanol vehicle 12 h post-transfection for 24 h. Transfected cells were washed once in phosphate-buffered saline and harvested in 1× reporter lysis buffer (Promega) as per the manufacturer's instructions. Luciferase activity was quantified in a reaction mixture containing 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 1 mM ATP, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, using an LB 9507 luminometer (EG & G Berthold) and 1 mM D-luciferin as substrate. The steroid receptor transcriptional activity is normalized to reporter activity in the absence of transfected steroid receptors and to protein concentration as determined by the Bradford protein assay (Bio-Rad). Since MCF-7 cells contain endogenous ER, transcriptional activity of the receptor is normalized to XETL activity in the presence of ER antagonist ICI 182,780. The data presented represent the average of two experimental values, with error bars representing the range of the data points.

Immunoblotting

To prepare protein extracts from transfected cells, whole cell extracts prepared for luciferase assay in 1× reporter lysis buffer were normalized for total protein and boiled for 3 min in SDS sample buffer. Protein extracts were fractionated by 12% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon membrane (Millipore Corp.), and probed with anti-ERα polyclonal antibody (HC-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Rho GDI polyclonal antibody (A-20; Santa Cruz Biotechnology), or anti-c-Myc monoclonal antibody (9E10; Santa Cruz Biotechnology). The blots were developed using horseradish peroxidase-coupled sheep anti-mouse or goat anti-rabbit antibodies and the ECL substrate as per the manufacturer's instructions (Amersham Pharmacia Biotech).

RESULTS

A Genetic Screen for Activators of ERα Transcriptional Enhancement—Concomitant serine to alanine mutations at N-terminal phosphorylation sites 104, 106, and 118 (ER_{AAA}) result in a ~50% reduction in ER transcriptional activity in mammalian cells (42, 43). To determine whether the transcriptional activity of ER_{AAA} is also reduced in yeast, strains were constructed containing a galactose-inducible expression vector encoding either WT ER or ER_{AAA} and an ER-responsive reporter plasmid. The transcriptional activities of WT ER and ER_{AAA} were measured as a function of hormone concentration. Compared with WT ER, ER_{AAA} exhibited ~40% reduction of

[2] D. Michaelson and M. Philips, unpublished results.

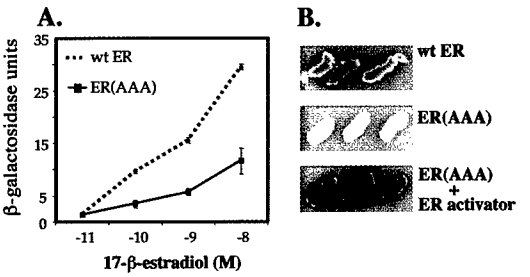

FIG. 1. **Isolation of yeast factors that increase transcriptional activation by ERα.** *A*, transcriptional activation of WT ER and ER_{AAA} as a function of 17β-estradiol concentration. Yeast strains were transformed with either a galactose-inducible WT ER or ER_{AAA}, along with an ERE-containing β-galactosidase reporter plasmid. Transcriptional activation by the WT ER (*dotted line*) and ER_{AAA} (*solid line*) in response to increasing 17β-estradiol concentration was determined by liquid β-galactosidase assay as described under "Experimental Procedures." Note that the ER_{AAA} in yeast exhibits ~40% of the WT ER transcriptional activity at each estradiol concentration tested. The dosage suppression screen was carried out in the presence of 1×10^{-10} M 17β-estradiol, conditions under which the ER_{AAA} phenotype is the most pronounced. *B*, the relative activity of WT ER, ER_{AAA}, and ER_{AAA} with an ER activator. Three independent colonies on X-gal indicator plates in the presence of 1×10^{-10} M 17β-estradiol are shown and represent WT ER with an empty expression vector (*WT ER*), ER_{AAA} plus an empty expression vector (*ER (AAA)*), and ER_{AAA} plus the RDI1 suppressor plasmid (*ER (AAA) + ER activator*). Under these conditions, colonies expressing WT ER are blue, ER_{AAA}-expressing colonies appear white, and ER_{AAA}-expressing the ER activator RDI1 are blue.

transcriptional activity at all hormone concentrations tested, suggesting that the ER_{AAA} is less efficient at engaging in the interactions necessary for transcriptional activation (Fig. 1*A*).

The ER_{AAA} phenotype is most striking at 0.1 nM 17β-estradiol. Under these conditions, yeast colonies expressing WT ER are blue, while ER_{AAA}-expressing colonies appear white (Fig. 1*B*). To screen for ER activators, yeasts expressing ER_{AAA}, along with an estrogen-responsive reporter gene, were transformed with a high copy yeast genomic library and assayed for receptor transcriptional activation on X-gal indicator plates containing 0.1 nM 17β-estradiol. Candidate high copy suppressors changing the ER_{AAA}-expressing yeast from white to blue were selected for further analysis (Fig. 1*B*). Of the 29,000 colonies screened, which represents approximately 3 times the size of the yeast genome, we identified six yeast open reading frames that enhance ER transcriptional activation (Table I).

A search of the yeast genome data base revealed that two of the candidate suppressors were yeast homologues of mammalian proteins previously shown to affect ER transactivation. These include 1) *CKA1* (44), a homologue of the mammalian α-subunit of casein kinase II that phosphorylates ER at serine 167 *in vitro* (45) and 2) *CAD1* (46), a member of the Jun transcription factor family that synergizes with ER in mammalian cells (47). In addition to genes known to regulate ER activity, several genes not known to affect ER were identified. *YAK1* (48), a serine/threonine kinase with homology to ANPK, a protein kinase that interacts with the zinc finger region of the AR and increases AR-dependent transcriptional activation, was isolated once (49). In addition, *MCK1* (50), a protein kinase with homology to glycogen synthase kinase-3 (51), was identified once. We also isolated *RDI1* (14), the yeast Rho guanine nucleotide dissociation inhibitor (Rho GDI), three times, and *LRG1* (52), a yeast protein that contains a GTPase-activating protein (GAP) homology domain, once. Although LRG1 is presently linked to GAP merely through sequence homology, it is interesting to note that GAP and RDI1 are both negative regulators of Rho GTPases. The recovery of known ER regulators together with the repeated isolation of certain genes indicates that the approach was sound and that the library was probably screened to saturation. Since Rho GDI negatively regulates Rho GTPases, this result suggests that the Rho GTPases may modulate ER transcriptional activation and is the focus of this report.

Rho GDI Expression Increases ER Transactivation—Among the human Rho GDIs, RDI1 is most similar to human Rho GDIα, which negatively regulates the best studied Rho GTPases, RhoA, Rac1, and Cdc42. To examine whether the mammalian Rho GDI affects ER transcription in mammalian cells, we tested the ability of human Rho GDIα to enhance ER transcriptional activity in the human osteosarcoma cell line U2OS. ER-negative U2OS cells were transiently transfected with ERα, an ER-responsive reporter plasmid, along with increasing amounts of Rho GDIα. As shown in Fig. 2*A*, Rho GDIα stimulates ER transactivation in a dose-dependent manner. Enhancement of ER transcriptional activation by Rho GDIα was also observed for ER_{AAA} mutant (not shown). To ensure that this enhanced transcriptional activity was not a result of increased ER protein production, we monitored protein expression in whole cell extracts using Western blot analysis. Fig. 2*B* illustrates that ER levels are not increased by Rho GDI expression. Indeed, the steady state concentration of ER decreased slightly with increasing Rho GDI expression, indicating that the effect of Rho GDIα on ER activity is greater than that observed. The effect of Rho GDI on ER transactivation is not restricted to single cell type, since Rho GDIα also enhanced ER transactivation in MCF-7 and Ishikawa cells (Fig. 2*C*). Thus, Rho GDIα can act as a positive regulator of ER-dependent transcriptional activation in a variety of mammalian cell lines.

Rho GDI Specifically Activates Steroid Hormone Receptors—We next tested the ability of Rho GDIα to affect transactivation by other members of the steroid receptor family, ERβ, GR, and AR, using transient transfection assays. Our results indicate that Rho GDIα also increased the transcriptional activity of ERβ, GR, and AR in a dose-dependent manner (Fig. 3, *A–C*). To determine whether Rho GDI-mediated activation is specific to steroid receptors, we tested the effect of Rho GDIα on Sp1- and SRF-dependent transactivation. Rho GTPase signaling has been previously shown to enhance transcriptional activation by SRF (18); thus, we would expect Rho GDIα, as a negative regulator of Rho GTPases, to decrease SRF transcriptional activity. Consistent with this idea, Rho GDIα expression decreased SRF activity from a reporter plasmid containing the c-Fos SRF element (Fig. 4*A*). Similarly, Sp1 transcriptional activity using an Sp1-responsive reporter also decreased in response to Rho GDI overexpression (Fig. 4*B*). Taken together, these results strongly suggest that Rho GDI specifically increases transactivation by steroid hormone receptors, perhaps through a mechanism involving suppression of Rho GTPase signaling.

Rho GTPases Inhibit ER Transactivation—The GTPases known to interact with Rho GDIα include RhoA, Rac1, and Cdc42. To determine whether Rho GDI increases ER transactivation by inhibiting the Rho GTPases, we expressed constitutively active forms of Rho GTPases (RhoA.V14, Rac1.L61, and Cdc42.L61) in U2OS cells and examined ER transcriptional activation. As shown in Fig. 5, expression of RhoA.V14, Rac1.L61, and Cdc42.L61 decreased ER transcriptional enhancement. Active forms of Rho GTPases also decreased ER transactivation in MCF-7 and Ishikawa cells (Fig. 6). In all three cell types, expression of the constitutively active forms of RhoA, Rac1, and Cdc42 resulted in an accumulation of filamentous actin, as determined by fluorescent phalloidin staining.[3] These results are consistent with the model that Rho GDI

[3] L. F. Su and M. J. Garabedian, unpublished observations.

TABLE I
Yeast genes that enhance ER transactivation

At the top are shown yeast homologues of two previously known mammalian regulators that affect ER function. At the bottom are four factors that appear to link ER transcriptional activation to signal transduction pathways previously not known to affect ER function.

Gene	Function	Mammalian homologue	Effect on ER	Times identified
CKA1	α subunit of casein kinase II	Casein kinase II	Phosphorylates ER S167 *in vitro*	1
CAD1	Yeast Jun family	c-Jun	Potentiates ER transactivation	1
YAK1	Ser/Thr kinase	ANPK	Enhances ER transactivation	1
MCK1	Ser/Thr/Tyr kinase	GSK-3	Enhances ER transactivation	1
RDI1	Rho GDP dissociation inhibitor	Rho GDIα	Enhances ER transactivation	3
LRG1	Contains LIM domains similar to Rho GTPase-activating proteins	Unknown	Enhances ER transactivation	1

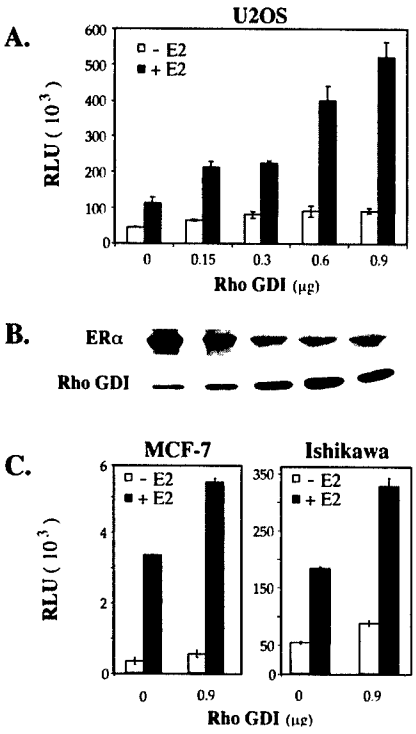

FIG. 2. **Enhancement of ERα transcriptional activation by overexpression of Rho GDIα.** *A*, ER-deficient U2OS cells (1.2×10^5 cells/35-mm dish) were transiently transfected using LipofectAMINE Plus reagent with 0.1 μg of ERα expression construct or empty vector, 0.2 μg of the ERE-containing reporter gene XETL, and increasing amounts of Rho GDIα, as indicated. 12 h after the transfection, cells were treated with 100 nM 17β-estradiol (*E2*) (*dark bars*) or the ethanol vehicle (*light bars*) for 24 h, harvested, and assayed for luciferase activity. ERα transcriptional activity is normalized to XETL reporter activity in the absence of ER. The data represent the mean of an experiment done in duplicate, which was repeated three times. *B*, expression of ERα does not increase by Rho GDIα coexpression. Whole cell extracts were prepared from transfected cells as described under "Experimental Procedures," and the expression of ERα and Rho GDIα was analyzed by Western blotting. *C*, MCF-7 and Ishikawa cells were transfected as above and assayed for luciferase activity. For Ishikawa cells, ERα transcriptional activity is normalized to XETL reporter activity in the absence of ER. For MCF-7 cells that contain endogenous ER, transcriptional activity of the receptor is normalized to XETL activity in the presence of ER antagonist ICI 182,780. The data represents the mean of experiments done in duplicate, which were repeated two times.

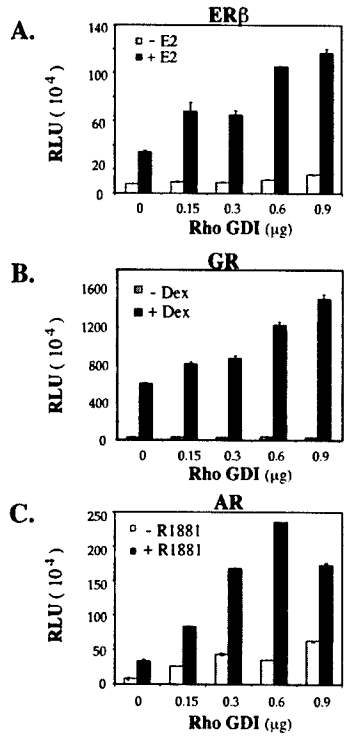

FIG. 3. **Rho GDIα enhances the transcriptional activation by ERβ, GR, and AR.** U2OS cells were transfected as described in Fig. 2 with paired expression and reporter plasmids for ERβ + XETL (*A*), GR + $XG_{46}TL$ (*B*), or AR + $XG_{46}TL$ (*C*) and, along with the indicated amount of Rho GDIα, were treated with 100 nM 17β-estradiol (*E2*), dexamethasone (*Dex*), and R1881, respectively, and harvested. In each case, receptor transcriptional activity shown is normalized to reporter activity in the absence of the receptor. The data shown represent experiments done in duplicate that have been repeated at least twice with similar results.

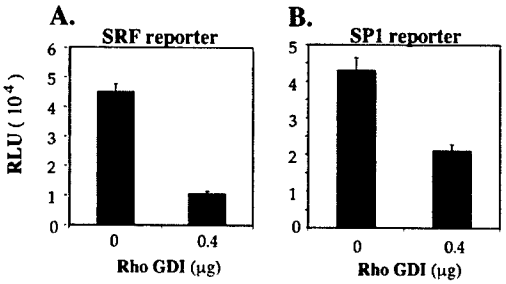

FIG. 4. **Rho GDI inhibits transcriptional activation by SRF and Sp1.** U2OS cells were transfected as in Fig. 2 with 0.4 μg of Rho GDIα together with 0.2 μg of SRF reporter (*A*) or Sp1 reporter (*B*), harvested after 24 h, and assayed for luciferase activity. Results shown represent an experiment done in duplicate and repeated twice.

activates ER transcriptional enhancement by antagonizing Rho GTPases.

As an independent means of examining the effect of RhoA inhibition on ER transcriptional activation, we ectopically expressed the *Clostridium botulinum* C3 transferase, a protein toxin that ADP-ribosylates and inhibits RhoA but not Rac1 or Cdc42 (37, 38). As with Rho GDI, expression of C3 transferase results in an enhancement of ER transcriptional activity but decreases SRF transcriptional activation in U2OS cells (Fig. 7*A* and data not shown). Inhibition of ER transcriptional activation by activated RhoA, but not Rac1 or Cdc42, was also relieved by C3 coexpression (not shown). Ectopic expression of C3

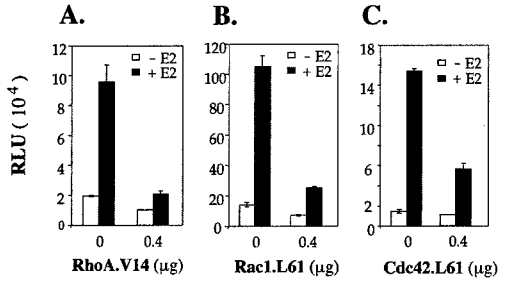

FIG. 5. **The Rho GTPases, RhoA, Rac1, and Cdc42, inhibit ER transcriptional activation.** U2OS cells were transfected as in Fig. 2 with the indicated amount of constitutively active forms of the Rho GTPases, RhoA.V14, Rac1.L61, and Cdc42.L61, along with 0.1 μg of ERα and 0.2 μg of XETL. Cells were treated with 100 nM 17β-estradiol (E2) 12 h post-transfection and harvested after 24 h of estradiol treatment. ER transcriptional activity as depicted is normalized to reporter activity in the absence of ER. Results shown represent an experiment done in duplicate and repeated twice with similar results.

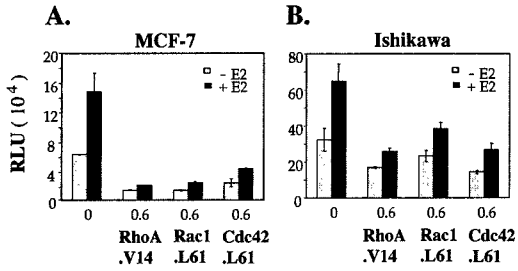

FIG. 6. **The Rho GTPases inhibit ER transcriptional activation in MCF-7 and Ishikawa cells.** MCF-7 and Ishikawa cells were transfected with the indicated amount of the constitutively active forms of the Rho GTPases, RhoA.V14, Rac1.L61, and Cdc42.L61, and ER transcriptional activity was measured as described in the legend to Fig. 2. Shown is a representative experiment performed in duplicate and repeated three times with similar results.

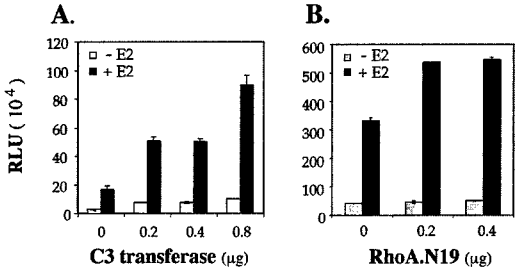

FIG. 7. **Inhibition of endogenous RhoA by C3 transferase and dominant negative RhoA potentiates ER transactivation.** U2OS cells were transfected as in Fig. 2 with the indicated amount of C3 expression vector (*A*) or dominant negative form of RhoA (RhoA.N19) (*B*) along with 0.1 μg of ERα and 0.2 μg of XETL. Cells were treated as described in the legend to Fig. 2, and ER transcriptional activation was measured. Shown is a representative experiment performed in duplicate.

transferase also increased ER transcriptional activation in MCF-7 and Ishikawa cells (not shown). Likewise, inhibition of endogenous RhoA by expression of a dominant negative form of RhoA (RhoA.N19) also results in greater ER transactivation (Fig. 7*B*). Thus, inhibition of RhoA results in enhanced ER transcriptional activation, indicating that Rho-mediated signaling events suppress ER transactivation.

DISCUSSION

We have demonstrated that Rho GDIα enhances the transcriptional activity of the ERα as well as ERβ, GR, and AR but not SRF or Sp1. We also show that activated mutant forms of RhoA, Rac1, and Cdc42 decrease, whereas inhibition of endogenous RhoA by C3 transferase or dominant negative RhoA increases ER transcriptional activation. From these results, we conclude that the enhanced ER transactivation observed upon Rho GDIα overexpression is mediated by antagonism of Rho GTPases and implicates the Rho family proteins RhoA, Rac1, and Cdc42 in signaling to ER.

What is the mechanism underlying the modulation of ER transactivation by RhoA? Since Rho GTPases mediate actin cytoskeleton reorganization as well as the activation of multiple signaling pathways, such as c-Jun N-terminal kinase (JNK) and p38, Rho-mediated inhibition of ER may result from either of these events. The ability of the Rho GTPase family members to repress ER transcriptional activity suggests that RhoA-, Rac1-, and Cdc42-mediated signaling to ER may converge at some common point through a shared signaling molecule. An attractive candidate for such a common regulator is LIM kinase (53). The GTP-bound forms of RhoA and Rac1/Cdc42 activate LIM kinase via phosphorylation through effector kinases ROCK and Pak, respectively (54, 55). The activated LIM kinase phosphorylates cofilin, an actin-binding protein, thereby inhibiting its actin-depolymerizing activity and leading to the accumulation of actin filaments. Recently, it has been shown that changes in the actin cytoskeleton can affect transcriptional activation by SRF (56). In a model reminiscent of that proposed for regulation of SRF by actin (56), we speculate that suppression of ER transactivation could result either from releasing an ER corepressor that is associated with free G-actin or from binding a coactivator to actin filaments, thus preventing its interaction with the ER. One such putative actin-regulated factor is the SWI/SNF complex, which has previously been shown to be a coactivator for steroid receptors, including ER, in both yeast and mammalian cells (57–59) and contains β-actin as well as two actin-related protein subunits (60–62). We are currently testing whether SWI/SNF and/or LIM kinase mediate the modulatory effects of Rho GTPases on ER transactivation. While the effect of actin cytoskeletal changes on ER remains unknown, actin dynamics may provide a means of modulating ER transcriptional activity during normal development or in pathological settings, such as tumor progression, when cells undergo extensive actin reorganization.

Alternatively, changes in ER transcriptional regulatory properties may result from the activation of signal transduction pathways by Rho GTPases. For example, Rac1/Cdc42 activate JNK and p38 mitogen-activated protein kinase pathways, which may affect ER or its coregulatory factors via phosphorylation. Unlike Rac1/Cdc42, RhoA is not thought to activate the JNK and p38 pathways; therefore, it is unlikely that the activated Rho GTPases are effecting ER transactivation via these pathways. Nevertheless, since we have not excluded the possibility that JNK and p38 are mediating the effect of the Rho GTPases on ER transcriptional activation, we are currently testing the impact of activation and inhibition of JNK and p38 on receptor transactivation.

A cellular activity induced by activated RhoA, Rac1, and Cdc42 is NF-κB, which has been shown to inhibit steroid receptor transactivation by forming inhibitory heterocomplexes (63, 64). It is tempting to speculate that the inhibition of ER by the Rho GTPases is mediated by NF-κB. However, our preliminary findings suggest that inhibition of NF-κB by overexpressing IκB does not relieve the repressive effects of Rho GTPases on ER transactivation (not shown), suggesting that Rho GTPases regulate ER independent of NF-κB.

Recently, an ER-interacting protein, termed Brx, was identified and shown to contain a domain virtually identical to the Rho GEF Lbc, although its enzymatic activity has not been demonstrated (26). Overexpression of Brx in Ishikawa cells increases ER transcriptional activation, and a dominant nega-

tive form of Cdc42, but not RhoA or Rac1, reduces its coactivator function (26). Our results differ from this report in assessing the effect of Rho GTPases on ER transactivation and showing that RhoA, Rac1, and Cdc42 negatively regulate ER transcriptional activity. This apparent discrepancy between what would be predicted from Rubino *et al.* (26), that Cdc42 increases ER transactivation, and our results showing that Cdc42, RhoA, and Rac1 decreased ER transcriptional activation may be attributed to methodological or cell-specific differences. Alternatively, since Brx coactivator function is probably mediated by direct ER binding, the inhibition of ER activity by the dominant negative form of Cdc42 may have resulted from competition between ER and dominant negative Cdc42 for Brx binding, rather than from blocking the signaling pathway downstream of Brx. In contrast, the effect of Rho GDI and the Rho GTPases on ER appears to be indirect. Localization studies indicate that ER and Rho GDI are found in distinct subcellular compartments, with Rho GDI residing in the cytoplasm, whereas ER is confined to the nucleus.[3] In addition, GST-Rho GDI is unable to associate with estradiol-bound ER, although it is capable of binding Rho A *in vitro*.[3] While Brx and Rho GTPases may modulate ER activity through distinct mechanisms, the identification of different components in the Rho signaling pathway as modulators of ER transactivation underscores their importance in receptor regulation.

Our findings suggest that the Rho GTPases decrease transcriptional activation by ERα, thus establishing a novel pathway of cross-talk between cell surface receptors that regulate Rho GTPase signaling and steroid receptor transcriptional activation. Another example of cross-talk between the cell surface and ER is the modulation of ER ligand-independent transcriptional activation by the epidermal growth factor/Ras/mitogen-activated protein kinase signaling pathway (65, 66). It has been shown that treatment of cells with epidermal growth factor results in ER ligand-independent activation and phosphorylation by the mitogen-activated protein kinase, Erk1 (31, 67). Although the mechanism of this increased ER transcriptional activation remains to be elucidated, it probably involves phosphorylation-dependent cofactor recruitment (68). Thus, Ras acts as a positive regulator of ER transcriptional enhancement (67),[3] whereas Rho GTPases suppress receptor transactivation. We speculate that the opposing actions of Ras and Rho GTPases on ER-mediated transcriptional activation provide a means of fine tuning the ER transcriptional response to changes in the extracellular environment.

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Regulation of GRIP1 and CPB coactivator activity by Rho GDI modulates estrogen receptor transcriptional enhancement

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Running title: Rho GDI regulates ER coactivators

Summary

Estrogen receptor alpha (ER) coordinates gene expression with cellular physiology in part by controlling receptor: cofactor interactions in response to extracellular signals. We have previously shown that the Rho signaling pathway modulates ER transcriptional enhancement. We now demonstrate that Rho GDI-dependent increase in ER transcriptional activation is dependent on the ER AF-2 coactivator binding site, prompting us to examine regulation of receptor coactivators by Rho GDI. Indeed, Rho GDI cooperates with GRIP1 to increase ER ligand-independent and ligand-dependent transcriptional activation, and also enhances GRIP1 transcriptional activity when GRIP1 is tethered to DNA. The GRIP1activation domain 1 (AD1), which binds CBP/p300, is necessary for Rho GDI to modulate GRIP1 activity. Using E1A to inhibit the endogenous CBP/p300 and a Gal4-CBP fusion protein to assay CBP activity, we find that the effect of Rho GDI on ER transcriptional activation is CBP/p300-dependent. Importantly, the ability of CBP/p300 to transduce the Rho GDI signal occurs through both GRIP1-dependent and -independent pathways. These data suggest a complex interplay between ER transcriptional regulation and the Rho signaling pathways through modulation of receptor cofactors, which may have evolved to coordinate receptor-dependent gene expression with Rho-regulated events, such as cell migration. We speculate that dysregulation of the Rho-ER axis may participate in cancer progression.

Introduction

The estrogen receptor alpha (ER) is a ligand-dependent transcription factor that is an important regulator of cell growth, differentiation, and malignant transformation. Transcriptional activation by ER is accomplished through specific and general cofactor complexes that assemble with the receptor at target promoters to regulate transcription. Although many cofactors have been described that participate in ER transcriptional regulation, the cellular signals and physiological contexts that modulate the activity of these cofactors are not well understood.

ER contains at least two transcription activation functions (AFs): constitutively active AF-1 in the N-terminus of the protein and ligand-dependent AF-2 at the ER C-terminus. These AFs represent surfaces capable of interaction with general transcription factors (GTFs) and additional transcriptional regulatory factors termed coactivators. Estradiol binding to the ER promotes a conformational change in the receptor and the formation of a surface for protein-protein contacts between AF-2 and coactivators. Among the numerous coactivators identified to date, one of the best characterized is the p160 family of proteins comprised of SRC1 (NcoA1), GRIP1 (TIF2, NcoA2), and RAC3 (ACTR, AIB1, P/CIP, TRAM). Although initial characterization of p160 proteins indicate that they participate in transcriptional activation by ER through AF-2 and interact with the receptor in a ligand-dependent manner (1-3), the p160s can also interact with ER N-terminal region and increase AF-1 transactivation independent of ligand (4). These interactions are mediated by two distinct regions on the p160s: the central NR-boxes bind ER AF-2 (5), whereas the p160 C-terminus interacts with the ER N-terminal A/B domain (4). The p160 proteins are believed to enhance ER transactivation by

recruiting other transcriptional regulatory factors through two activation domains, AD1 and AD2. AD1 interacts with CREB binding protein (CBP) and p300 (6), whereas AD2 has been shown to associate with the coactivator-associated arginine methyltransferase 1 (CARM1) and the p68/72 family of proteins, which also bind to the ER A/B domain (7,8). ER recruits CBP/p300 both indirectly, through an association with the p160 proteins, as well as directly, through the ER A/B domain, thereby increasing AF-1 activity and facilitating the interaction between ER AF-1 and AF-2 (9). CBP and p300 are multifunctional proteins that stimulate ER transcriptional activation by interacting with components of the basal transcriptional machinery such as RNA Pol II, TBP, and $TF_{II}B$, and facilitate an association with other transcription factors and coregulators such as p/CAF (10,11). Additionally, CBP/p300 contain a histone acetyltransferase (HAT) activity that acetylates both histone and non-histone proteins, which, interestingly, include ER and the p160s (12,13). Thus, a complex picture of signal transduction by ER is emerging that appears to rely on a collaboration of multiple factors for regulation of gene expression.

We have previously demonstrated that ER transcriptional activation is increased by overexpression of Rho guanine nucleotide dissociation inhibitor alpha (Rho GDI) and this effect is mediated through an inhibition of Rho GTPases (14). Rho GDI is a cytoplasmic protein that acts as a negative regulator of Rho GTPases, including RhoA, Rac1 and Cdc42, by blocking the dissociation of GDP (15). In addition, Rho GDI controls subcellular localization of the Rho GTPases through binding the C-terminal isoprenoid modification, thus preventing their insertion into the plasma membrane and

modulating the ratio of the active membrane-bound and inactive soluble forms of Rho proteins (16).

In this report we analyze the mechanism by which Rho GDI increases ER transcriptional activity. We determine the contribution of the ER transcriptional activation domains and examine the activity of p160 and CBP/p300 receptor cofactors by Rho GDI. Our results indicate that the Rho GDI signal is transduced to ER by CBP/p300 through GRIP1-dependent and -independent pathways.

Experimental Procedures

Plasmids

The ER reporter plasmid XETL contains one ERE from the Xenopus vitellogenin A2 gene, upstream of the herpes simplex virus thymidine kinase (tk) promoter (-109) linked to the firefly luciferase coding sequence. The Gal4 reporter plasmid, p5xGal4tk-luciferase was a generous gift from Naoko Tanese. The human ER containing constructs pcDNA3-wtER and pcDNA3-ER_{AAA} with S104, S106, and S118 mutated to alanine have been previously described (17). pCDNA3-ER_{2L} contains full length ER with leucine to alanine mutations at amino acids 539 and 540. ER1-269 was created by substituting NotI/BamHI fragment of Gal4-AB with amino acids 64-269 from pRK5-ER_{1-269}, and contains Gal4-DBD fusion to sequence 1-269 encoding AF-1 and the DNA binding domain. $ER_{282-595}$ containing C-terminal ER fragment from amino acids 282 to 595 fused to Gal4-DBD was a gift from Paul Webb. ER_{AF2} expresses a larger ER C-terminal fragment, extending from amino acids 179 to 595 that includes sequences encoding ER DNA binding domain and AF-2 was a gift from Donald McDonnell. The expression construct pCDNA3-Rho GDIα has been described previously (14). The p160 expression vectors: pcDNA3-GRIP1, pCR3-hSRC1A and pCMX-FlagRAC3 were generously provided by Inez Rogatsky and Keith Yamamoto. Plasmids encoding various GRIP1 derivatives fused to the Gal4 DBD were a gift from Michael Stallcup: pM-GRIP1.FL contains GRIP1 $_{5-1462}$ subcloned into EcoRI and SalI sites of pMvo; pM-GRIP1.ΔAD1, contains GRIP1 $_{5-1462}$ with amino acids 1057-1108 deleted, pM-GRIP1.ΔAD2, comprises a GRIP1 fragment encoding amino acids 5-1121, and pM-GRIP1.ΔAD1/2,

harbors GRIP1 $_{5\text{-}1121}$, lacking amino acids 1057-1108. pCDNA3-GRIP1.ΔAD1 was created by exchanging the XhoI/XbaI fragment of pCDNA3-GRIP1.FL with C-terminal sequence of pM-GRIP1.ΔAD1. Gal-CBPc containing Gal4 DBD fused to CBP C-terminal fragment 1678-2441 was a gift from Rosalie Uht. The E1A expression construct, pCI-HA-E1A-12S, was generously provided by Matt Paulson and David Levy. For each transfection, pCMV-LacZ plasmid produced β-galactosidase and was used as an internal control for transfection efficiency.

Cell culture, transfection, and luciferase assays

Human osteosarcoma U2OS (HTB 96) cell line was obtained from American Type Culture Collection. Cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 10 units/ml each of penicillin and streptomycin (Cellgro), and 2 mM L-glutamine (Cellgro). 2 x 10^5 cells were seeded onto 35-mm plates in phenol red-free DMEM (Cellgro) supplemented with 10% charcoal-stripped fetal bovine serum and 2 mM L-glutamine. Transfections using Lipofectamine Plus reagent (Invitrogen) were performed according to manufacturer's recommendation. Total amount of DNA transfected is held constant in each transfection using the corresponding empty vector. Cells were treated with 100 nM 17β-estradiol, 100 nM 4-OH-tamoxifen, or ethanol vehicle 12 hr post-transfection for 24 hr. If no treatment is indicated, cells were harvested 36 h after transfection. Transfected cells were washed once in phosphate-buffered saline and harvested in 1X reporter lysis buffer (Promega) as per the manufacturer's instructions. Luciferase activity was quantified in a reaction mixture

containing 25 mM glycylglycine pH 7.8, 15 mM MgSO4, 1 mM ATP, 0.1 mg/ml BSA, 1 mM DTT, using a LB 9507 luminometer (EG&G Berthold) and 1 mM D-luciferin as substrate. The transcriptional activity for ER and Gal4 DNA-binding domain fusion proteins are normalized to reporter activity in the absence of transfected transcriptional activators, β-galactosidase activity as an internal control for transfection efficiency, and to protein concentration as determined by the Bradford protein assay (Bio-Rad).

Immunoblotting

To prepare protein extracts from transfected cells, cells were lysed with 1X sample buffer, containing 2%SDS, 0.1 M DTT, and 60 mM Tris pH 6.8. Whole cell extract was normalized for total protein, boiled for 5 min in SDS sample buffer and fractionated on either 8% or 12% SDS-polyacrylamide gel electrophoresis, for detection of GRIP1 and Rho GDI, respectively. Gel-fractionated proteins were then transferred to Immobilon membrane (Millipore), and probed with anti-ERα polyclonal antibody (HC-20, Santa Cruz Biotechnology), anti-GRIP1 polyclonal antibody (PA1-846, Affinity Bioreagents), or anti-Rho GDIα polyclonal antibody (A-20, Santa Cruz Biotechnology). The blots were developed using horseradish peroxidase-coupled goat anti-rabbit antibodies and the Enhanced Chemiluminescence (ECL) substrate as per the manufacturer's instructions (Amersham Pharmacia Biotech).

Results

Rho GDI increases ER transcriptional activation via AF-2-dependent and -independent mechanisms

To understand how Rho GDI regulates ER transcriptional enhancement, we first examined whether the activities of AF-1 or AF-2 were modulated by Rho GDI expression. The contribution of each of these two transcriptional activation functions can be distinguished by using tamoxifen, which binds the ER LBD and alters the conformation of AF-2 such that coactivator association is disrupted. We transiently transfected U2OS cells with ER and Rho GDI expression vectors, and assessed ER transcriptional activation in the absence of ligand and in the presence of estradiol and tamoxifen on an ER-responsive reporter plasmid. ER activation by Rho GDI is roughly 7-fold upon estradiol stimulation and only 2-fold in the presence of tamoxifen (Fig. 1A; compare lanes 5 and 6 to 9 and 10), suggesting that ER AF-2 mediates the majority, but not all of Rho GDI-dependent ER activation.

To further examine the role of AF-2 in mediating Rho GDI signaling, we used an ER derivative defective in p160 association. This mutant ER (ER_{2L}) contains twin leucine to alanine mutations at amino acid 539 and 540, which are located on helix 12 that forms a part of the p160 binding surface. It has been previously shown that these alterations decrease coactivator association with ER (18), without affecting ligand- or DNA-binding (19). In the absence of ligand treatment, we observed a 7-fold increase in wt ER transcriptional activity by expression of Rho GDI, but only a 2-fold increase on ER_{2L} (Fig. 1A; compare lanes 1 and 2 to 3 and 4). Likewise, in the presence of estradiol, Rho GDI is less effective at increasing ER_{2L} activity as compared to wt ER (Figure 1A,

lanes 5-8). The ability of specific mutations in coactivator binding site to disrupt Rho GDI-mediated increase in ER transcriptional activation supports the hypothesis that Rho GDI regulate ER largely by modulating ER AF-2 activity, implicating alterations in p160 binding or activity. Interestingly, Rho GDI does appear to regulate ER in an AF-2 independent manner, as neither tamoxifen treatment, AF-2 disruption by mutagenesis (ER_{2L}), nor a combination of both completely abolish Rho GDI-dependent increase in ER transcriptional activity (Fig. 1A, lanes 9-12). Taken together, these data suggest that induction of ER transcriptional activation by Rho GDI is mediated largely by an ER AF-2-dependent and to a lesser extent an AF2-independent mechanism.

To further examine the effect of Rho GDI on ER activation domains, we tested whether Rho GDI can regulate AF-1 or AF-2 independently using ER fragments containing either AF-1 (ER_{1-269}) or AF-2 ($ER_{282-595}$) fused to Gal4 DNA binding domain. As shown in Figure 1B, Rho GDI increases the transcriptional activity of ER AF-2 in a ligand-dependent manner roughly 5-fold, providing further support for Rho GDI as an activator of ER AF-2. Additionally, Rho GDI increases the transcriptional activity of ER_{1-269} about 2-fold, consistent with AF-2-independent ER induction by Rho GDI in the presence of tamoxifen or mutations in the AF-2 coactivator binding site.

Rho GDI and GRIP1 enhance ER transcriptional activation cooperatively

Since a majority of Rho GDI-mediated increase in ER transcriptional activation is dependent on AF-2, which binds p160 coactivators, we examined the relationship between Rho GDI and the p160 coactivator GRIP1. As shown in Figure 2A, in the absence of hormone, ER transcriptional activation is increased 3-fold by overexpressed

Rho GDI, 3-fold by GRIP1, but is increased 22-fold when Rho GDI is coexpressed with GRIP1. Similarly, Rho GDI and GRIP1 together induce a 15-fold increase in ER transcriptional activation upon estradiol treatment. This increase in ER transcriptional activation is not the result of elevated ER protein expression (Fig. 2A, bottom panel) and ER nuclear localization is not affected by GDI or GRIP1 expression (not shown).

To determine if transcriptional synergy between GRIP1 and Rho GDI is a feature unique to GRIP1, we tested the effect of Rho GDI with the other members of the p160 family, SRC1 and RAC3. SRC1 also cooperates with Rho GDI to increase ER transcriptional activation, although the fold induction of SRC1 with Rho GDI on ER transcriptional activation is less pronounced as compared to GRIP1, with only a 3-fold increase in the absence of ligand, and a 6-fold enhancement upon estradiol treatment (Fig. 2B). In contrast, we were unable to demonstrate a significant increase in ER activation by RAC3 and Rho GDI over that of Rho GDI alone (Fig. 2C). These differences may reflect functional diversity between GRIP1, SRC1, and RAC3 or different levels of p160 expression. Since in our system Rho GDI cooperated most strongly with GRIP1, we have focused our efforts on characterizing the effect of Rho GDI on GRIP1.

Rho GDI increases GRIP1 transcriptional activity in an AD1-dependent manner

To determine the mechanism by which Rho GDI and GRIP1 increase ER transcriptional activation, we first examined whether Rho GDI increases GRIP1 transcriptional activity. Although GRIP1 is not a sequence-specific transcription factor, it contains two activation domains whose activity can be monitored by tethering GRIP1

to DNA via a heterologous DNA binding domain. Rho GDI increased the transcriptional activity of GRIP1 fused to the Gal4-DBD (Gal4-GRIP1.FL, Fig. 3A), roughly 3-fold (Fig. 3B), suggesting that Rho GDI is an upstream regulator of GRIP1, which amplifies its coactivation potential, thereby increasing ER transcriptional activation. These results also suggest that it is the activity of GRIP1, rather than GRIP1 binding to ER, that is stimulated by Rho GDI.

We next mapped the GRIP1 domain mediating the effect of Rho GDI on ER using GRIP1 derivatives lacking AD1 (Gal4-GRIP1ΔAD1), AD2 (Gal4-GRIP1ΔAD2), or AD1/2 (Gal4-GRIP1ΔAD1/2) (Fig. 3A). Our results indicate that deletion of AD1 or AD1/2 abolishes the Rho GDI-dependent increase in GRIP1 activity, whereas deletion of AD2 does not (Fig. 3C). Although deletion of AD2 resulted in a large increase in GRIP1 activity, the activity of this derivative was further augmented by Rho GDI expression. This increase in GRIP1.ΔAD2 has not been explored. The expression of GRIP1 variants was confirmed by immunoblot analysis (Fig. 3C, bottom panel). Taken together, these results suggest that Rho GDI regulates GRIP1 transcriptional activation, and that this requires the CBP/p300-interacting region, AD1.

Rho GDI regulates CBP transcriptional activity

Since Rho GDI-responsive region of GRIP1 maps to the CBP/p300-binding domain, Rho GDI may increase GRIP1 activity by regulating CBP/p300 function. To determine if CBP/p300 is a downstream target of Rho GDI, we asked whether Rho GDI regulates the transcriptional activity of CBP. CBP-dependent transcriptional activation was assayed by cotransfecting Gal4-CBP fusion protein with a Gal4 responsive reporter,

with or without Rho GDI. Indeed, Rho GDI increases transcriptional activity of CBP in a dose-dependent manner (Fig. 4A).

The viral oncoprotein E1A has been shown to associate with HAT domain and the neighboring CH3 region of CBP and p300, thereby inhibiting their HAT-dependent and -independent activities (10,20,21). We cotransfected a Gal4 fusion of full length GRIP1 with or without Rho GDI, and in the presence or absence of E1A-12S. As shown in Figure 4B, E1A decreases GRIP1 activity and completely blocks stimulation of GRIP1 transcriptional activation by Rho GDI, suggesting that CBP/p300 are required for Rho GDI to increase GRIP1 activity. The activity of a Gal4-GRIP1.ΔAD1derivative which lacks the CBP/p300-interacting region , is not blocked by ectopic E1A expression, suggesting that the inhibitory effect of E1A on GRIP1-dependent transcriptional activation is specific for CBP/p300 (not shown). These findings are consistent with our results that GRIP1's CBP/p300-interacting AD1 domain mediates the effect of Rho GDI on GRIP1. Furthermore, these results suggest that CBP/p300, in addition to GRIP1, maybe largely responsible for mediating the increase in ER transcriptional activation induced by Rho GDI.

CBP/p300 are required for Rho GDI to increase ER transcriptional activation

We next examined whether CBP is required for Rho GDI to increase ER transcriptional activation, by cotransfecting ER and Rho GDI with E1A. Figure 5A shows that inhibition of CBP/p300 by E1A inhibits ER transcriptional activity, consistent with the role of CBP/p300 as being important ER regulators in U2OS cells. Importantly, E1A also abolishes Rho GDI-mediated increase in ER transcriptional activation without

decreasing the level of ER or Rho GDI protein expression (Fig. 5A; bottom panels). If CBP/p300 are required to transduce Rho GDI signaling to ER via GRIP1, we anticipate that E1A will decrease the cooperativity with respect to ER transcriptional activation. Indeed, the addition of E1A reduced ER transcriptional activation (Fig. 5B, compare lanes 1 and 5) and blocked the synergistic increase in ER activity induced by coexpression of Rho GDI and GRIP1 (Fig. 5B, compare lanes 4 and 8). Since CBP and p300 are inhibited by E1A, these results indicate that Rho GDI modulates ER activity through an E1A-sensitive step that most likely requires the activity of CBP/p300.

Increased ER transcriptional activation is partially dependent on recruitment of CBP/p300 by GRIP1

Although inhibition of Rho GDI/GRIP1 synergy by E1A supports a model whereby Rho GDI activates ER through a CBP/p300 dependent step, it does not address whether recruitment of CBP/p300 to GRIP1 is important in this process. As CBP can also interact with ER directly, independent of p160 proteins, it is conceivable that the synergistic enhancement of ER transcriptional activation by Rho GDI and GRIP1 results from CBP/p300 both increasing ER transcriptional activation directly, as well as indirectly through an increase in GRIP1 activity. We examined the ability of GRIP1.ΔAD1 to increase ER transcriptional activation synergistically with Rho GDI. Recall that the AD1 region of GRIP1 binds CBP/p300 and deletion of AD1 has been shown to abolish CBP/p300-mediated increase of GRIP1 function (22). Here, we show that GRIP1.ΔAD1, lacking a CBP/p300 binding site, is still able to enhance ER transcriptional activation in the presence of Rho GDI (Fig. 6A). Thus, recruitment of

CBP/p300 by GRIP1 only partly accounts for the ER activation by Rho GDI in combination with GRIP1. It is likely that CBP/p300 transduces Rho GDI signaling by binding and coactivating ER through parallel pathways involving both GRIP1 AD1-dependent and -independent mechanisms.

To more closely examine the GRIP1 AD1-independent activity, we tested whether E1A is able to repress it, thereby implicating CBP/p300 in Rho GDI and GRIP1.ΔAD1 cooperativity. In agreement with previous experiments, co-transfection of Rho GDI and GRIP1 results in an increase in ER transcriptional activation that is abolished by E1A expression (Fig. 6B). Likewise, Rho GDI collaborates with GRIP1.ΔAD1, albeit at a reduced level. Importantly, this cooperative ER activation by Rho GDI and GRIP1.ΔAD1 is fully repressed by E1A, suggesting that CBP/p300 mediates GRIP1 AD1-independent ER activation by Rho GDI.

ER activation by Rho GDI and GRIP1 requires both receptor activation domains and is independent of AF-1 phosphorylation

We next examined the mechanism by which Rho GDI and GRIP1 cooperate to increase ER transcriptional activation. p300 has been previously shown to bind and stimulate transcriptional activity of both ER AF-1 and AF-2, as well as increase ligand-induced interaction between these two activation domains (9). Since Rho GDI activates CBP/p300, and CBP/p300 also appear to be essential for cooperativity between Rho GDI and GRIP1, we speculated that ER activation by Rho GDI and GRIP1 may reflect a collaboration between ER AF-1 and AF-2. This hypothesis was tested using a truncated receptor containing the DNA binding domain and AF-2, but not AF-1, extending from

amino acid 179 to 545 (ER_{AF-2}). As shown in Figure 7B, deletion of the AF-1 domain decreases ER transcriptional activation and impairs Rho GDI-mediated increase in ER activity, suggesting that AF-1 contributes to the total ER transcriptional activity in U2OS cells, and to ER activation by Rho GDI. In contrast, AF-2 appears to be sufficient for ER induction by GRIP1, which increases ER-AF-2 activity by 5-fold, relative to a 4-fold induction on full length ER. Importantly, no synergy between Rho GDI and GRIP1 is observed on ER-AF-2, suggesting that AF-1 domain plays an essential role in mediating cooperative ER activation by Rho GDI and GRIP1. This notion is further substantiated by the fact that the transcriptional activity of an ER-2L mutant, which disrupts of AF-2, is still increased in response to Rho GDI and GRIP1 coexpression, albeit to a lesser degree than wt ER (Fig. 7B). Together, these data suggest that induction of ER transcriptional activation by Rho GDI and GRIP1 relies on the cooperative actions of ER AF-1- and AF-2.

Since AF-1 phosphorylation sites at S104, S106, and S118 are important for AF-1 activity, we next addressed the role of AF-1 phosphorylation in mediating cooperativity between Rho GDI and GRIP1. Using full length ER with serine to alanine mutation at S104, S106, and S118 (ER_{AAA}), we demonstrate that Rho GDI alone or in combination with GRIP1, is able to increase ER_{AAA} activity comparable to its effect on wt ER (Fig. 7B). Thus, although AF-1 domain is important for Rho GDI to induce ER transcriptional activation, Rho GDI action on ER is not mediated by the AF-1 phosphorylation sites.

Discussion

We provide evidence that Rho GDI increases ER transcriptional activation by regulating the transcriptional activity of both AF-1 and AF-2 through the coactivators GRIP1 and CBP/p300. Several lines of evidence suggest the cooperative induction of ER by Rho GDI and GRIP1 is mediated by CBP/p300. First, this cooperativity requires AD1, the CBP/p300 interacting domain of GRIP1. Second, Rho GDI stimulates the transcriptional activity of CBP tethered to DNA. Third, inhibition of endogenous CBP/p300 by the viral oncoprotein E1A blocks the increase in ER transcriptional activity by Rho GDI, as well as cooperativity between Rho GDI and GRIP1 on ER transcriptional activation. While E1A also binds a component of the mammalian Mediator complex, hSur2 (23,24), which is a subunit of DRIP/TRAP complex that binds nuclear receptors and stimulate ER transcriptional activation (25), it is unlikely that inhibition of this complex results in the E1A-mediated inhibition of ER transcriptional activation, since the E1A-12S isoform used is the alternative spliced product that does not interact with hSur2. Rather, we suggest that the Rho GDI-dependent increase in ER transcriptional activation is through an E1A-sensitive step that most likely involves CBP/p300.

How might Rho GDI increase ER transcriptional activation cooperatively with GRIP1? We speculate that Rho GDI increases GRIP1 transcriptional activity through enhancement of CBP/p300 binding or activity. However, because a GRIP1 derivative that no longer binds CBP/p300 (GRIP1.ΔAD1) retains some activity, Rho GDI may also cooperate with GRIP1 via an AD1-independent mechanism. This mechanism, nevertheless, appears to involve CBP/p300 since cooperative ER activation by Rho GDI and GRIP1.ΔAD1 is E1A-sensitive. Taken together, our data suggest that the Rho GDI

enhances ER transcriptional activation by stimulating CBP/p300 action, which, in turn increases ER transcriptional activation via parallel GRIP AD1-dependent and -independent mechanisms (Fig. 8). This result is consistent with recent findings from the Kraus lab that demonstrate CBP/p300 interactions with GRIP1 are required for ER transcription initiation *in vitro* (26).

Our initial studies suggested that Rho GDI increases ER transcriptional activation largely by regulating ER AF-2, as inhibition of AF-2 activity either pharmacologically using tamoxifen or with ER mutations, greatly diminishes the effect of Rho GDI on ER (Fig. 1). However, in light of the observation that AF-1 deletion abolishes synergistic increase in ER transcriptional activation by Rho GDI and GRIP1 (Fig. 7), it appears that ER AF-1 plays an essential role in permitting Rho GDI and GRIP1 to cooperatively enhance ER transcriptional activity. This requirement for ER AF-1 is also consistent with ligand-independent induction of ER by Rho GDI and GRIP (Fig. 2A). Thus, both ER AF-1 and AF-2 are necessary, but individually not sufficient for the cooperative effect of Rho GDI and GRIP1 on ER transcription activation. We suggest that the synergistic increase in ER activity most likely reflects collaboration between ER AF-1 and AF-2, with both contributing to overall transcriptional enhancement, but with AF-2 being more important in coactivator recruitment. Our data are consistent with a model whereby Rho GDI overexpression increases the number of CBP/p300 recruited to ER, either directly or indirectly through GRIP1 binding, thereby enhancing the functional interaction between AF-1 and AF-2 (Fig. 8).

While the AF-1 domain is required for Rho GDI to induce ER transcriptional activation, Rho GDI action on ER does not require S104, S106, and S118

phosphorylation sites (Fig. 7B). Thus, direct phosphorylation of S104 and S106 by cyclinA/Cdk2 (17,27), and of S118 by MAPK (28) or cyclinH/Cdk7 (29) is unlikely to mediate Rho GDI-dependent increase in ER transcriptional activity. However, MAPK activation and cell cycle regulation by Rho signaling pathway may still contribute to changes in ER transcriptional activation via phosphorylation and modulation of coactivator function. Indeed, recent studies have shown that the p160 coactivators are modified by MAPK signaling (30-32). CBP/p300 are also targets for MAPK phosphorylation, which appears to stimulates HAT activity (33-36). As MAPKs regulate p160 and p300/CBP activity, a link between the MAPK pathway and Rho GDI-dependent increase in ER transcriptional activation appears plausible. Alternatively, GRIP1 and CBP/p300 may be regulated by other common effector of Rho signaling pathway, such as the PAK family of serine/threonine kinases.

Rho signaling has been implicated in transcriptional regulation of a handful of transcription factors, including as SRF and NF-κB (37,38). We report here that Rho GDI targets CBP/p300 and increases CBP transcriptional activity. Since CBP and p300 modulate the activity of a large number of transcription factors, induction of CBP/p300 activity by Rho GDI could result in wide spread changes in gene expression. Thus, it is possible that the role of Rho signaling in regulating gene transcription is currently under-appreciated, with many more transcription factors responsive to Rho GDI still to be identified. With respect to ER transcriptional activation, modulation of CBP/p300 and GRIP1 activity by Rho signaling pathways provide an additional regulatory input to modulate ER transcriptional activity in response to extracellular signaling.

Although the overall consequences of ER activation by Rho GDI is currently unknown, the interplay between Rho signaling and ER function may prove particularly important during normal development when regulation of cellular proliferation by ER may need to be coordinated with Rho-regulated events, such as cellular migration. Dysregulation of the Rho-ER axis may uncouple this regulation, thereby contributing to cancer progression. Activation of ER is an early mitogenic event in breast cancer, however, it has also been suggested that the receptor may restrict tumor progression by inhibiting cell invasion and metastasis. For example, introduction of ER into an ER-negative metastatic breast cancer cell line results in reduced invasiveness *in vitro* and metastatic tumor formation *in vivo* (39). Similarly, MCF-7 cells with a high ER content display decreased motility *in vitro* (40). ER-expressing breast tumors are less assertive and invasive with a more favorable disease outcome, whereas ER-negative tumors are typically more aggressive and metastatic, and are associated with a worse prognosis. Thus, although ER appears to promote cellular proliferation, loss of ER results in a more aggressive tumor phenotype.

Interestingly, while ER expression is decreased in advanced breast tumors, the level of Rho GTPases increases with the degree of tumor progression (41). Indeed, overexpression of RhoC is sufficient to stimulate invasion of melanoma cells and is overexpressed in a particularly aggressive type of breast cancer prone to early metastasis (42), whereas the dominant negative RhoA represses the invasiveness of melanoma cells (43).

The opposing effects of Rho GTPases and ER on cell invasion is consistent with a model where Rho GTPases inhibit ER transcriptional activity, thereby blocking ER target

genes that result in the suppression of cell migration. In contrast, Rho GDI overexpression would promote expression of ER target genes that restrain cell invasion by inhibiting Rho GTPases. It would be interesting to examine the effect of Rho signaling pathway on endogenous genes regulated by ER, and determine whether Rho GDI specifically regulates genes involved in cell migration and invasion that correlates with breast tumor progression.

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Figure legends

Figure 1. **Contribution of the ER transcriptional activation domains to Rho GDI-dependent ER transcriptional activation**

A) Tamoxifen and ER p160 binding mutant ER_{2L} disrupt Rho GDI-dependent increase in ER transcriptional activation. ER-deficient U2OS cells (2 x10^5 cells/35 mm dish) were transiently transfected using Lipofectamine Plus reagent with 0.2 μg of the ERE-containing reporter plasmid XETL, 0.1 μg of wt ER or ER_{2L} expression construct, along with 0.6 μg of Rho GDIα where indicated. Total amount of DNA was equalized with the corresponding empty vector. Twelve hr after the transfection, cells were treated with ethanol vehicle (ETOH), 100 nM 17β-estradiol (Estradiol) or 100 nM 4-OH-tamoxifen (Tamoxifen) for 24 hr, harvested, and assayed for luciferase activity. ER transcriptional activity was normalized to XETL reporter activity in the absence of ER. B) Rho GDI increases the transcriptional activity of ER AF-1 and AF-2. U2OS cells were transfected as above with Gal4-ER $_{1\text{-}269}$ (also see inset) or Gal4-ER $_{282\text{-}595}$, along with 0.2 μg of p5xGal4tk-luciferase reporter and 0.6 μg of Rho GDIα where indicated. Cells treatment and reporter activity assays were performed as described above. Results shown represent a single experiment done in duplicate with the error bars representing the range of the mean. The experiment was repeated three times with similar results.

Figure 2 **Cooperative enhancement of ER transcriptional activity by Rho GDI and GRIP1**

A) U2OS cells were transfected as in Figure 1 with 0.2 μg of XETL reporter, 0.1 μg of ER, and either vector only (white bar), 0.6 μg of Rho GDI (light gray bar), 0.6 μg of

GRIP1 (dark gray bar), or 0.6 μg Rho GDI and 0.6 μg GRIP1 (black bar). Cells treatment and ER transcriptional activation assays were performed as described in Figure 1. Shown is a representative experiment performed in duplicate, with the error bars representing the range of the mean. Whole cell extracts were prepared from transfected cells as described in the "Experimental Procedures", and the expression of ERα was analyzed by Western blotting (bottom panel). B) and C) Effects of the p160 cofactors on Rho GDI-dependent ER transcriptional enhancement. Cells were transfected as above except that SRC1 or RAC3 was substituted for GRIP1and assayed for luciferase activity. The data represent the mean of an experiment done in duplicate, which was repeated at least three times with similar results.

Figure 3 **Rho GDI increases transcriptional activity through GRIP1 AD1**

A) Schematic representation of GRIP1 expression constructs fused to Gal4 DNA binding domain. GRIP1 contains an N-terminal bHLH/PAS domain, three NR boxes and two activation domains: AD1 binds CBP/p300 and AD2 interacts with CARM1 and p68/72. B) Rho GDI increases transcriptional activity of GRIP1. U2OS cells were transfected with 0.2 μg of p5xGal4tk-luciferase reporter, 1.0 μg of Gal4-GRIP1.FL, and indicated amount of Rho GDIα. Cells were harvested and luciferase activity was measured 36 h post transfection. C) AD1 is required for Rho GDI-dependent increase in GRIP1 activity. U2OS cells were transfected with 1.0 μg of either Gal4-GRIP1.FL, Gal4-GRIP1.ΔAD1, Gal4-GRIP1.ΔAD2, or Gal4-GRIP1.ΔAD1/2 as indicated and luciferase activity was determined as above. Whole cell extracts were prepared from transfected cells and the expression of GRIP1 was analyzed by immunoblotting using anti-GRIP1 antibody

(bottom panel).

Figure 4 **CBP is a target of Rho GDI**

A) Rho GDI increases CBP transcriptional activity. U2OS cells were transfected as in Figure 1 with 0.5 μg of Gal4-CBPc, 0.2 μg of p5xGal4tk-luciferase reporter, and indicated amount of Rho GDI. Cells were harvested and luciferase activity was measured 36 h post transfection. B) E1A blocks the Rho GDI-dependent increase in GRIP1 transcriptional activity. U2OS cells were transfected as above with 1.0 μg of Gal4-GRIP1.FL, 0.2 μg of p5xGal4tk-luciferase reporter, and indicated amount of Rho GDI in the absence (-) or presence (E1A) of 80 ng of pCI-HA-E1A-12S. Luciferase activity was assayed 36 h post transfection. Shown is a representative of three independent experiments. Error bars represent the range of the mean.

Figure 5 **Rho GDI-mediated increase in ER transcriptional activation is E1A-sensitive**

A) U2OS cells were transfected with 0.2 μg of XETL reporter, 0.5 μg of ER, along with the indicated amount of Rho GDI and E1A-12S. Cells were treated with 100 nM 17β-estradiol and luciferase activity was measured as in Figure 1. Whole cell extracts were prepared from transfected cells and the expression of ER and Rho GDI was analyzed by immunoblotting using anti-ERα and anti-Rho GDIα antibodies (bottom panels). B) E1A blocks the increase in ER transcriptional activation by Rho GDI and GRIP1. U2OS cells were transfected as in Figure 1 with 0.2 μg of XETL reporter, 0.1 μg of ERα, along with the either 0.6 μg of empty expression vector only (lanes 1and 5), Rho GDI (lanes 2 and

6), GRIP1 (lane 3 and 7), or Rho GDI and GRIP1 (lanes 4 and 8) in the absence or presence of E1A-12S (80 ng/dish; lanes 5 and 6 or 400 ng/dish; lanes 7 and 8). Cells were treated as described in Figure 1 and ER transcriptional activation was measured. Data shown are the mean of a representative experiment performed in duplicate and repeated three times with similar results. Error bars represent the range of the mean.

Figure 6 **Cooperative enhancement of ER transcriptional activity by Rho GDI and GRIP1 is impaired by deletion of CBP/p300 interaction domain of GRIP1 and abolished by E1A**

A) Cooperativity between Rho GDI and GRIP1 is partially dependent on the CBP/p300 binding domain of GRIP1. U2OS cells were transfected as in Figure 1 with 0.2 μg of XETL reporter and 0.1 μg of ER along with either 0.6 μg of Rho GDI (+) or 0.6 μg of GRIP1 (FL or ΔAD1, as indicated), or the combination of the two. Cells were treated with 100 nM 17β-estradiol and ER transcriptional activation was measured as described in Figure 1. B) E1A inhibits ER activation by Rho GDI and GRIP1. U2OS cells were transfected as above with the 0.6 μg of Rho GDI and 1.0 μg of GRIP1.FL or GRIP1.ΔAD1, as indicated, in the absence or presence of E1A. Experiment shown was performed in duplicate and repeated three times. The error bars represent the range of the mean.

Figure 7 **ER AF-1 is required for cooperativity by Rho GDI and GRIP1 and is independent of ER AF-1 phosphorylation**

A) Schematic of ER deletion and substitution constructs. ER_{AAA} contains serine to alanine mutations at three phosphorylation sites in AF-1 (serines 104, 106 and 118) that are phosphorylated by MAPK and cell cycle-regulated kinases. $ER_{AF\text{-}2}$ encompasses the DNA binding domain and AF-2, but lacks the A/B domain containing AF-1. The ER_{2L} mutant contains leucine to alanine mutations at amino acid 539 and 540 and disrupts the AF-2 coactivator binding surface. B) Cooperativity between Rho GDI and GRIP1 relies on ER AF-1. U2OS cells were transfected as in Figure 1 with either vector only (white bar) 0.6 μg of Rho GDI (light gray bar), 1.0 μg of GRIP1 (dark gray bar), or Rho GDI and GRIP1 (black bar), along with 0.2 μg of XETL reporter and 0.1 μg of the ER derivative indicated. Cells were treated with 100 nM 17β-estradiol and ER transcriptional activation was measured as described in Figure 1. Result shown is the mean of one experiment done in duplicate and repeated three times, where the error bars represent the range of the mean.

Figure 8 **Model for Rho GDI-dependent increase in ER transcriptional activation by GRIP1 and CBP**

Rho GDI stimulates the transcriptional activity of CBP/p300. CBP/p300 in turn, regulates ER both directly and indirectly by stimulating the activity of another coactivator, GRIP1. Cooperativity by Rho GDI and GRIP1 likely reflects greater recruitment of CBP/p300 via association with the ER AF-1, AF-2, and GRIP1.

Supplemental Figure 1 **Specificity of E1A action: Inhibition of GRIP1 transcriptional activation by E1A is abolished by deletion of CBP/p300 interaction region of GRIP1**

U2OS cells were transfected with 1.0 μg of Gal4-GRIP1 (full length [FL] or ΔAD1), along with 0.2 μg of p5xGal4tk-luciferase reporter and Rho GDI in the absence (-) or presence (E1A) of pCI-HA-E1A-12S. Luciferase activity was assayed 36 h post transfection. Shown is a representative of three independent experiments. Error bars represent the range of the mean.

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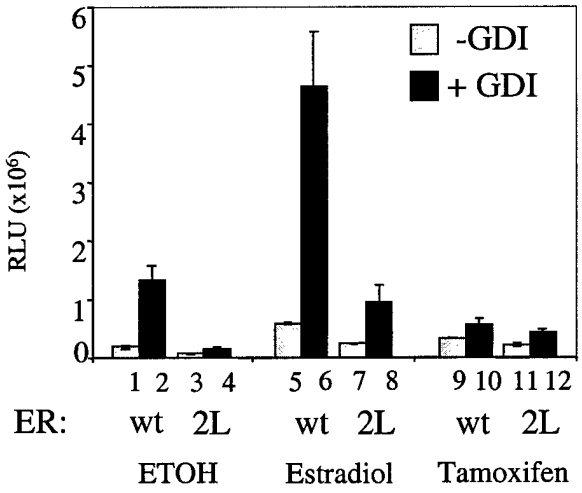
Figure 1

A


-GDI
+ GDI
RLU (x10⁶)
0
1
2
3
4
5
6
1 2 3 4 5 6 7 8 9 10 11 12
ER: wt 2L wt 2L wt 2L
ETOH
Estradiol
Tamoxifen

B

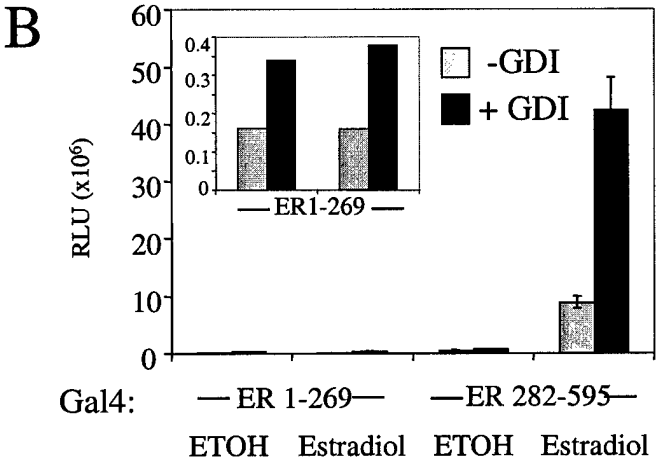

-GDI
+ GDI
RLU (x10⁶)
0
10
20
30
40
50
60
0.4
0.3
0.2
0.1
0
— ER1-269 —
Gal4: — ER 1-269— —ER 282-595—
ETOH Estradiol ETOH Estradiol

Figure 2

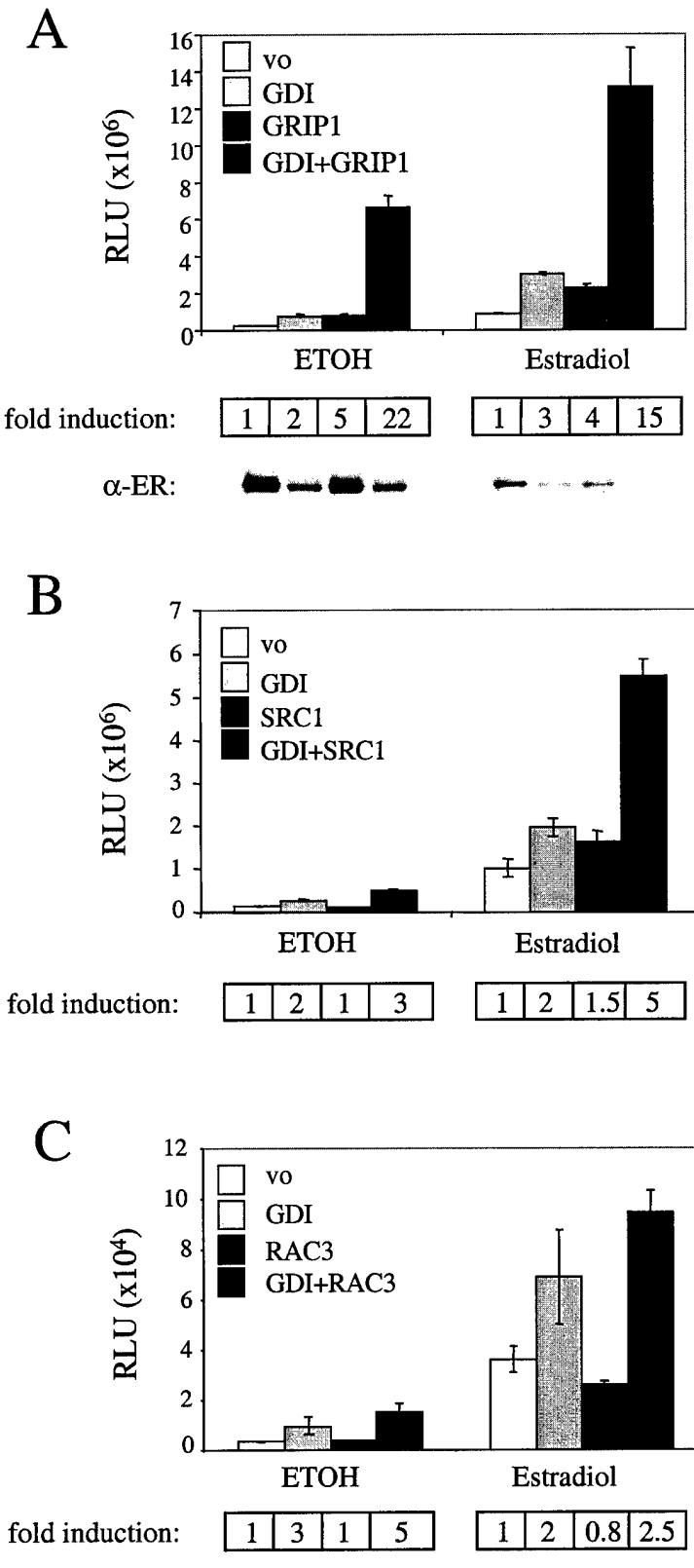

A
RLU (x10^6)
vo
GDI
GRIP1
GDI+GRIP1
ETOH
Estradiol
fold induction:
1 2 5 22
1 3 4 15
α-ER:
B
RLU (x10^6)
vo
GDI
SRC1
GDI+SRC1
ETOH
Estradiol
fold induction:
1 2 1 3
1 2 1.5 5
C
RLU (x10^4)
vo
GDI
RAC3
GDI+RAC3
ETOH
Estradiol
fold induction:
1 3 1 5
1 2 0.8 2.5

Figure 3

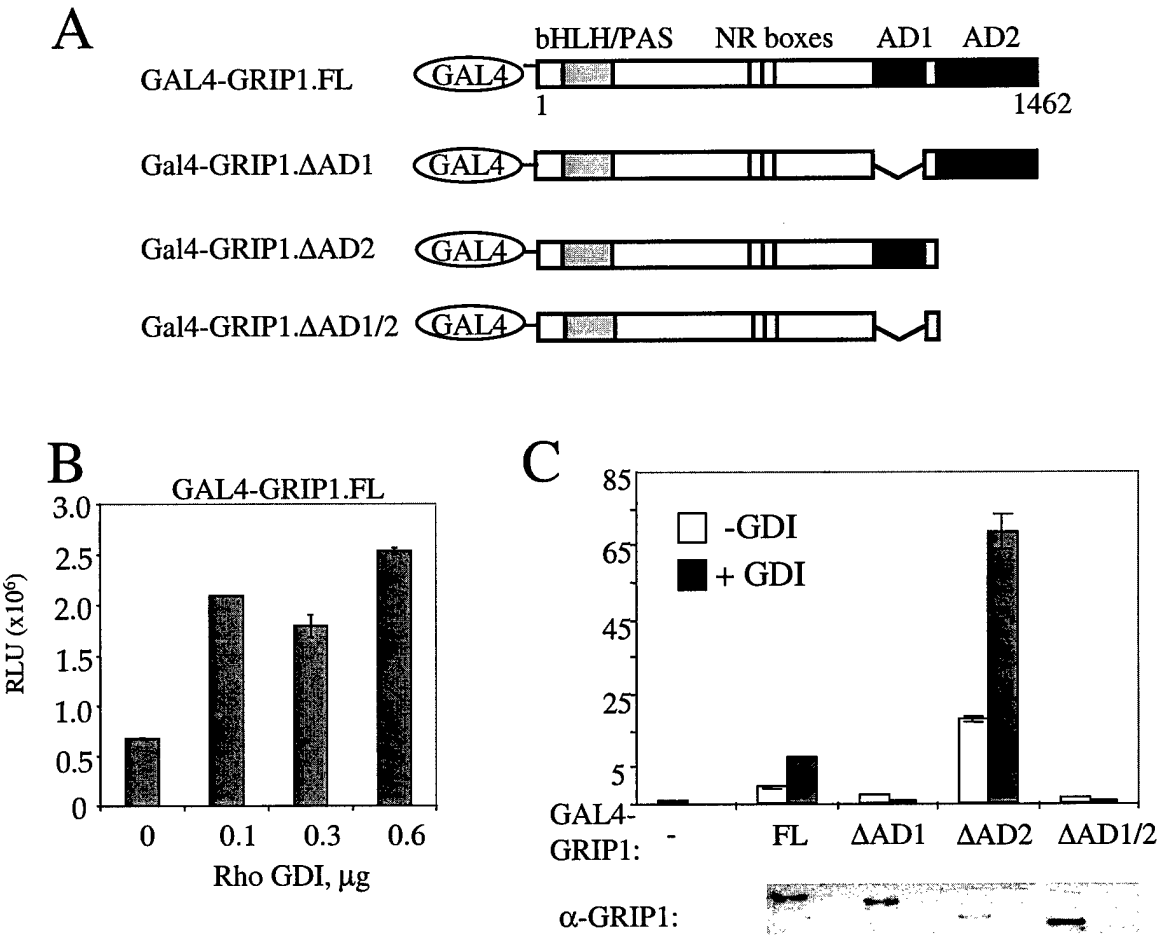
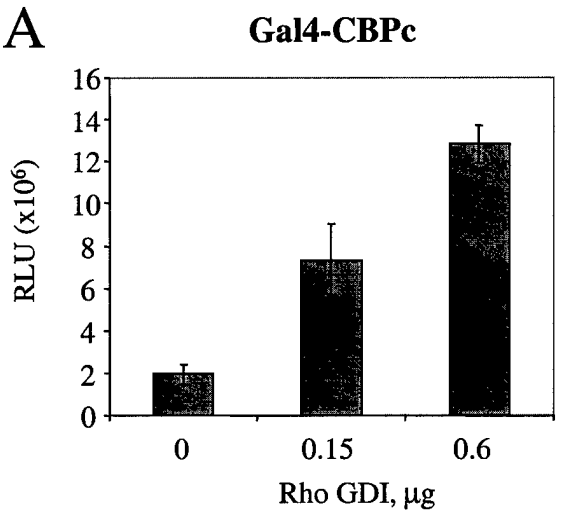

A
bHLH/PAS
NR boxes
AD1
AD2
GAL4-GRIP1.FL
GAL4
1
1462
Gal4-GRIP1.ΔAD1
Gal4-GRIP1.ΔAD2
Gal4-GRIP1.ΔAD1/2
B
GAL4-GRIP1.FL
RLU (x10⁶)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
0.1
0.3
0.6
Rho GDI, μg
C
85
65
45
25
5
-GDI
+ GDI
GAL4-GRIP1:
-
FL
ΔAD1
ΔAD2
ΔAD1/2
α-GRIP1:

Figure 4


A
Gal4-CBPc
RLU (x10⁶)
16
14
12
10
8
6
4
2
0
0
0.15
0.6
Rho GDI, µg

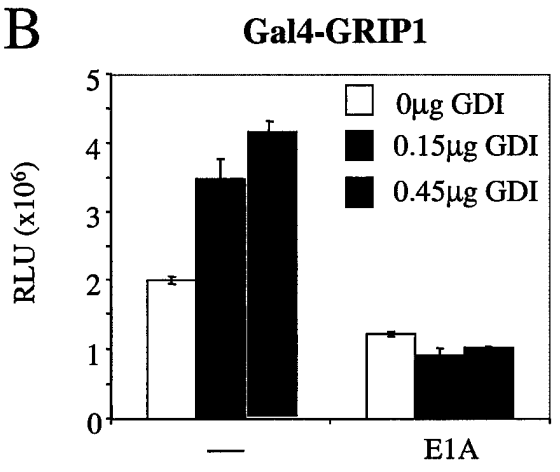
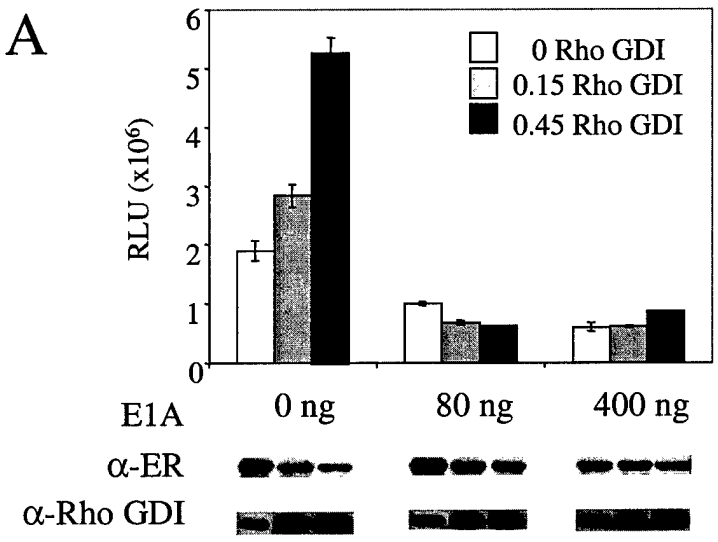

B
Gal4-GRIP1
RLU (x10⁶)
5
4
3
2
1
0
0µg GDI
0.15µg GDI
0.45µg GDI
—
E1A

Figure 5


A
RLU (x10⁶)
0 Rho GDI
0.15 Rho GDI
0.45 Rho GDI
E1A
0 ng
80 ng
400 ng
α-ER
α-Rho GDI

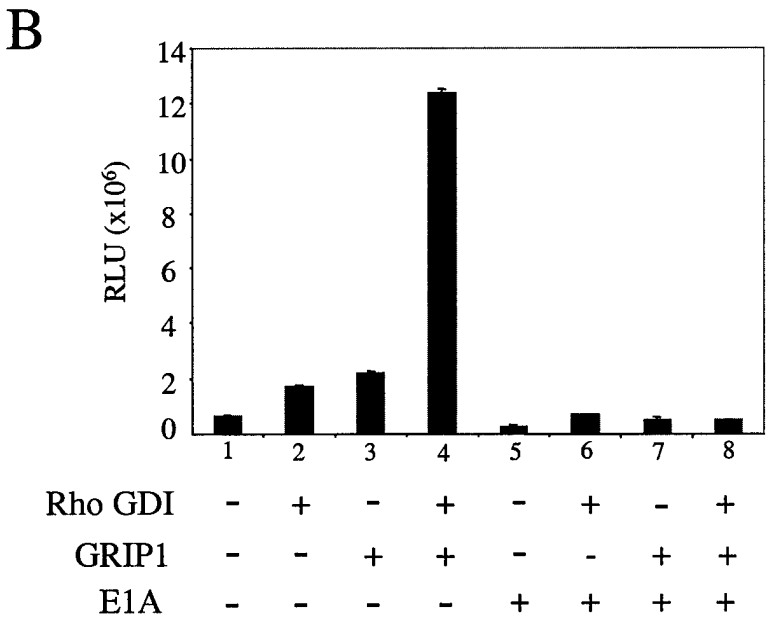
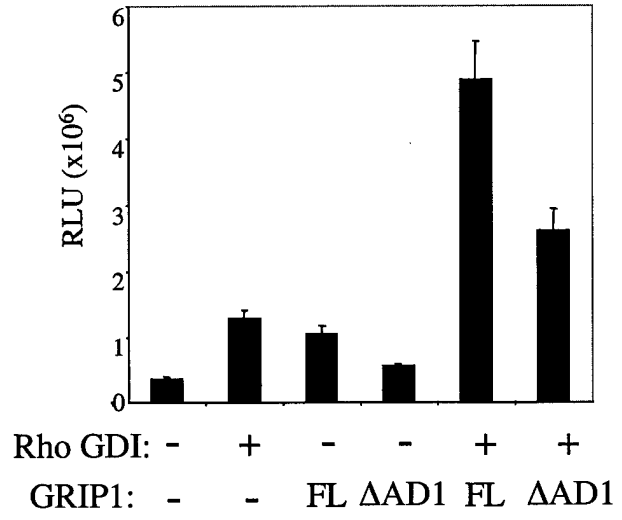

B
RLU (x10⁶)
Rho GDI
GRIP1
E1A

Figure 6

A


RLU (x10⁶)
0
1
2
3
4
5
6
Rho GDI: - + - - + +
GRIP1: - - FL ΔAD1 FL ΔAD1

B

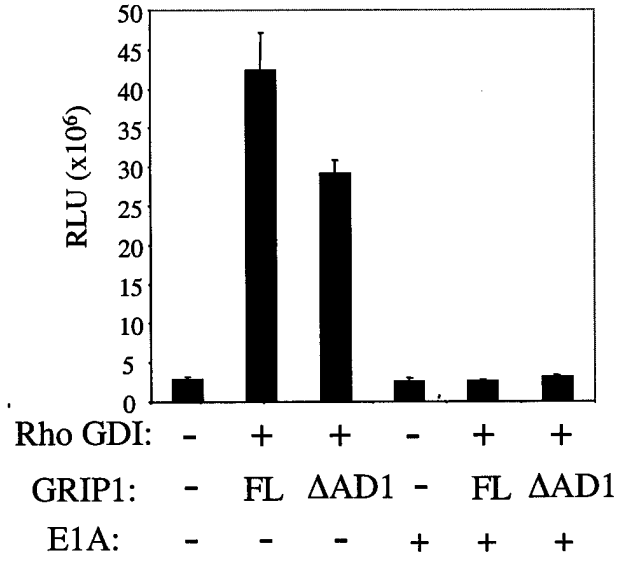

RLU (x10⁶)
0
5
10
15
20
25
30
35
40
45
50
Rho GDI: - + + - + +
GRIP1: - FL ΔAD1 - FL ΔAD1
E1A: - - - + + +

Figure 7

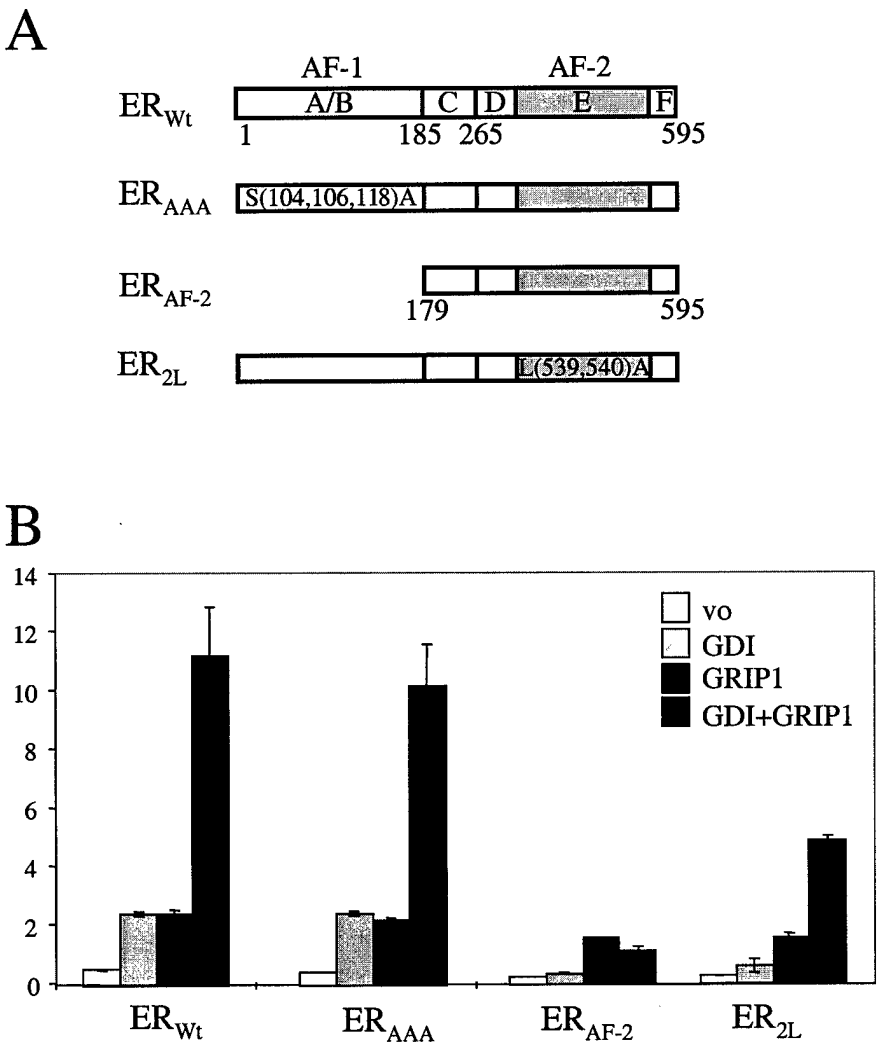
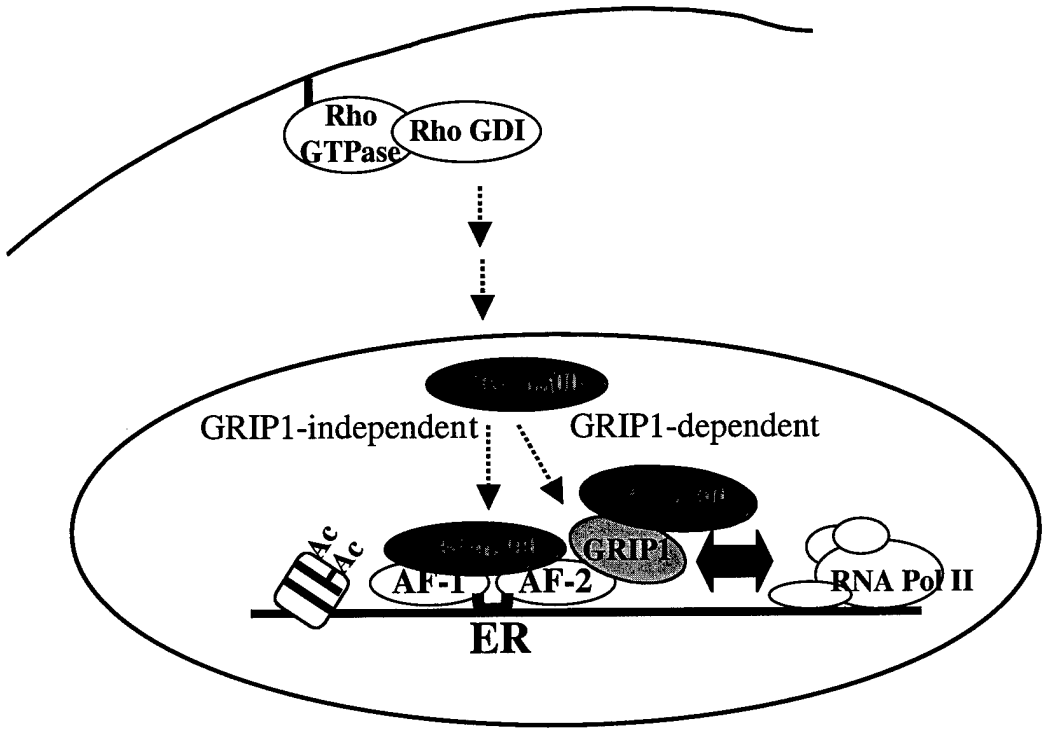

A
AF-1
AF-2
ER$_{Wt}$
A/B
C
D
E
F
1
185
265
595
ER$_{AAA}$
S(104,106,118)A
ER$_{AF-2}$
179
595
ER$_{2L}$
L(539,540)A
B
RLU (x10^7)
14
12
10
8
6
4
2
0
vo
GDI
GRIP1
GDI+GRIP1
ER$_{Wt}$
ER$_{AAA}$
ER$_{AF-2}$
ER$_{2L}$

Figure 8


Rho GTPase
Rho GDI
GRIP1-independent
GRIP1-dependent
Ac
Ac
AF-1
AF-2
GRIP1
RNA Pol II
ER

Supplemental Figure 1